

## **Remarks**

### **I. Claim Objections**

Applicants thank the Examiner for the indication of allowable subject matter, and have amended the claims to conform to his indication of allowable subject matter. Applicants solicit an indication that the claims are in allowable form in the subsequent Office Action.

### **II. Rejection of Claims 1-2 and 10-22 under 35 U.S.C. § 112, First Paragraph: Written Description**

Claims 79-81, 91, 96, 97, 100, 102, 103, 106, 108, 109, 112, 114, 115, 118, 121, 124, and 126 stand rejected under 35 U.S.C. § 112, first paragraph, as allegedly containing subject matter that was not described in the specification in a manner that reasonably conveys to one of ordinary skill in the art that the inventors had possession of the claimed invention at the time of filing.

Applicants disagree. Compliance with written description is essentially a fact-based inquiry. *See Enzo Biochem, Inc., v. Gen-Probe Inc.*, 296 F.3d 1316, 1324 (Fed. Cir. 2002) (citing *Vas-Cath Inc. v. Mahurkar*, 935 F.2d 1555, 1563 (Fed. Cir. 1991) and *In re DiLeon*, 436 F.2d 1404, 1405 (C.C.P.A. 1971)). Disclosure of “such descriptive means as words, structures, figures, diagrams formulas, etc., that fully set forth the claimed invention” satisfies the written description requirement. *See, Enzo Biochem*, 296 F.3d at 1329 (quoting from *Lockwood v. American Airlines, Inc.*, 107 F.3d 1565, 1571 (Fed. Cir. 1997)). Adequate written description of genetic material “requires a precise definition, such as by structure, formula, chemical name, or physical properties.” *Regents of the University of California v. Eli Lilly &*

*Co.*, 119 F.3d 1559, 1566 (Fed.Cir. 1997), *Fiers v. Revel*, 984 F.2d 1164, 1171 (Fed.Cir. 1993)).

The Examiner has maintained the outstanding written description rejection and has alleged that Applicants' arguments presented in the response dated May 27, 2003 were not persuasive because "the specification must provide some link between structure and function under the guidelines of 35 U.S.C. 101, which applicants have disclosed as fragments retaining translational control activity over the subject TIGR protein."<sup>1</sup> The Examiner asserts that what is at issue is "whether adequate description exists for fragments that retain the correlated activity."<sup>2</sup> The Examiner alleges that "fragments of 15 to 250 nucleotides that are expected to retain translational control over said [TIGR] protein have not been sufficiently described in the specification."<sup>3</sup> In addition, the Examiner alleges that there is a failure of the specification to provide some link between structure and function of the fragments retaining translational control activity over the subject TIGR protein.<sup>4</sup>

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<sup>1</sup> Applicants note that the Examiner has improperly relied upon 35 U.S.C. § 101 in constructing the rejection under § 112, first paragraph, based upon the "guidelines" for 35 U.S.C. § 101, which are neither statute, nor substantive rules. The utility requirement of 35 U.S.C. § 101 and the written description requirement of § 112, first paragraph are separate and distinct statutory requirements. In addition, as the Examiner has changed the scope and nature of the present rejection by interjecting a new statutory basis of rejection, the finality or the Office Action is improper and should be withdrawn.

<sup>2</sup> Applicants thank the Examiner for his acknowledgement that the disclosure sets forth SEQ ID NO.: 3, and that it is possible to envision fragments of SEQ ID NO.: 3 [from 15 to 250 nucleotides]. Office Action dated August 13, 2003 at page 4.

<sup>3</sup> Office Action dated August 13, 2003 at page 4.

<sup>4</sup> *See Id.* at 3.

Applicants respectfully disagree. Applicants have described the claimed fragments of SEQ ID NO.: 3 by supplying the sequence of SEQ ID NO.: 3, which is sufficient to meet the written description requirement.<sup>5</sup> The disclosure at page 27 states:

A functional regulatory region of the TIGR gene may be a TIGR promoter sequence. ... A number of regulatory elements are discussed below, and the equivalent of those activities can represent the functional regulatory region of the TIGR gene.

A functional regulatory region, as defined, does not need to be a complete and functional promoter that retains “translational control activity.” Moreover, the disclosure sets forth a substantial number of *cis* regulatory elements within SEQ ID NO.: 3, and sufficient information for the skilled artisan to recognize their function.<sup>6</sup>

Applicants respectfully disagree with the Examiner’s assertions that such fragments of the TIGR promoter “have only been described in terms of their function (of modulating translation), along with a method of obtaining (from the 6000 nucleotide SEQ ID NO:3),” and that “although it is possible to envision fragments of SEQ ID NO: 3 as alleged by applicants, it is not possible to envision such fragments having the broadly claimed function of ‘regulatory activity’.”<sup>7</sup> This is not the case. The disclosure sets forth a substantial number of *cis* elements within the claimed sequences including the claimed fragments, and more than sufficient information for a skilled artisan to recognize these elements and “to provide some link between structure and function” under 35 U.S.C. §

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<sup>5</sup> See, Applicants’ response filed May 27, 2003, which is herein incorporated by reference. The Examiner admits the disclosure sets forth SEQ ID NO.: 3, and that it is possible to envision fragments of SEQ ID NO.: 3 [from 15 to 250 nucleotides]. See, n. 2, *supra*.

<sup>6</sup> See *e.g.*, the disclosure at p. 27-35.

<sup>7</sup> See, the Office Action dated August 13, 2003 at p. 4.

101.<sup>8</sup> As such, the specification provides more than a sequence described by function and a method of obtaining such a sequence. Moreover, Applicants submit that their disclosure of the promoter sequence and *cis* elements therein is sufficient to place a person of skill in the art in possession of the claimed fragments, meeting not only the written description requirement, but also the enablement requirement § 112, first paragraph. In addition, a skilled artisan would be able to identify functional regulatory elements within the claimed sequences through a variety of techniques including the use of enhancer traps.<sup>9</sup>

In view of the foregoing, Applicants submit that they have met the written description requirements of § 112.<sup>10</sup>

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<sup>8</sup> Applicants maintain that there is no statutory basis for imposing this requirement on patentability. *See n. 1 supra*.

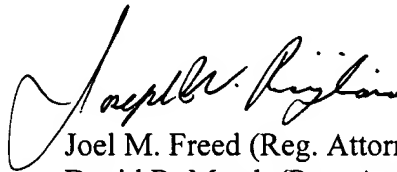
<sup>9</sup> Enhancer traps are described as “a recombinant construction containing a selectable marker driven by a promoter that has been weakened by removal of enhancers so that the transfected trap is only expressed if it comes under the influence of an endogenous enhancer. *See e.g.*, “Functional Identification of Genes Up- and Down-Regulated by Glucocorticoids in AtT-20 Pituitary Cells Using an Enhancer Trap,” Harrison, R. W. and Miller, J.C., *Endocrinology* 137(7), 2758-65 (1996), attached as Appendix A.

<sup>10</sup> Applicants additionally submit that they have provided more than sufficient disclosure to meet the enablement requirement of § 112.

### Conclusion

Applicants submit that the claims are in condition for allowance and solicit a notice of allowability at the earliest possible time. Should the Examiner have any questions regarding this application, the Examiner is encouraged to contact Applicants' undersigned representative at 202-942-5174.

Respectfully submitted,



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# Functional Identification of Genes Up- and Down-Regulated by Glucocorticoids in AtT-20 Pituitary Cells Using an Enhancer Trap\*

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## ABSTRACT

The AtT-20/D1 mouse pituitary tumor cell line has been used to study glucocorticoid regulation of POMC. We have used an enhancer trap to determine whether other glucocorticoid-regulated genes exist in AtT-20 cells. An enhancer trap is a recombinant construction containing a selectable marker driven by a promoter that has been weakened by removal of its enhancers so that the transfected trap is only expressed if it comes under the influence of an endogenous enhancer. For a selectable marker, we used a fusion gene coding for hygromycin phosphotransferase (Hy) and herpes simplex thymidine kinase. Thus, expression of this gene conferred hygromycin resistance and ganciclovir sensitivity. Suppression resulted in ganciclovir resistance and hygromycin sensitivity. An enhancerless promoter was produced using a truncated, transcriptionally inactive, form of the POMC promoter. AtT-20/D1 cells were transfected with this construct and cultured in medium containing hygromycin to kill any cells not expressing the Hy gene. The survivors were cultured in medium containing ganciclovir and dexamethasone and cloned. Clones in which the transgene was down-regulated by dexamethasone survived and were designated AtT-20/NET (for negative enhancer trap). Northern blot analysis confirmed that the transgene was down-reg-

ulated by dexamethasone as expected and that in at least one instance, suppression of the transgene was more complete than suppression of the full-length POMC promoter. Southern blot analysis after restriction enzyme digestion showed that each cell clone contained a single copy of the transgene, and PCR analysis of the promoter region showed that insertion had occurred in two unique sites in at least two cell clones. Another plasmid construct was prepared that contained the selectable gene but lacked any promoter elements. After transfection of AtT-20 cells with this vector, up-regulated enhancers were trapped by selection in hygromycin and dexamethasone followed by ganciclovir alone and designated AtT-20/PET cells (for positive enhancer trap). Up-regulation of the selectable gene in AtT-20/PET cells was confirmed by Northern blot analysis of dexamethasone-treated cells. In summary, glucocorticoid-regulated enhancers have been identified in AtT-20/D1 cells by an enhancer trap strategy that uses sequential selection under conditions that test whether the transgene is active. These results indicate that in addition to the well characterized, down-regulated POMC gene, there are other glucocorticoid-regulated genes in AtT-20/D1 cells that are both up-regulated and down-regulated by glucocorticoids. (*Endocrinology* 137: 2758-2765, 1996)

**G**LUCCORTICIDS affect the growth and differentiated function of cells from tissues as diverse as the immune, nervous, musculoskeletal, and pituitary systems (8, 33, 37, 47). These effects are mediated by the glucocorticoid receptor, a 90-kDa protein that functions as a transcription factor when charged with an active steroid (26, 50, 51). The activated receptor binds to specific DNA sequences, usually located in the gene's promoter region, and this interaction results in increased or decreased promoter activity. Thus, glucocorticoid-regulated genes have specific DNA structures that function as positive or negative transcription enhancers, whereas nonregulated genes do not. Theoretically and practically, the glucocorticoid-regulated genes of a cell determine its response to glucocorticoid stimulation.

Glucocorticoid regulation of corticotropin secretion by the anterior pituitary is a physiologically important process. The AtT-20 mouse pituitary tumor cell line has been extensively studied to determine how corticotropin is produced from its

precursor POMC and how glucocorticoids regulate this process. Processing of POMC to corticotropin and other peptides has been detailed (3, 5, 11, 46), and many components of the glucocorticoid regulatory mechanism have been analyzed. For instance, the glucocorticoid receptor contained in AtT-20 cells has been extensively characterized (14, 18, 19, 21, 23, 28, 32, 52-54), and its interaction with the POMC gene promoter has been described (34, 48). The intricate path of corticotropin synthesis and its tight control by glucocorticoids have led to speculation that the enzymes that process POMC to corticotropin might also be glucocorticoid regulated. However, there have been relatively few successful attempts to identify other glucocorticoid-regulated genes in AtT-20 cells.

We have used an enhancer trap strategy to produce AtT-20/D1 clonal cell lines in which glucocorticoid up- and down-regulated enhancers have been functionally identified. An enhancer trap is a transgene containing a selectable element, such as hygromycin phosphotransferase, that lacks a functional promoter due to deletion of the promoter's enhancers. Thus, the enhancer trap must integrate into chromosomal DNA near an endogenous enhancer to achieve expression. This strategy has been widely used in *Drosophila* (24, 44, 45) and has also been applied to mammalian cells (6, 12, 15, 27, 35). Evidence suggests that the transfected DNA integrates preferentially near the CpG-rich regions of ac-

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tively transcribed genes (31). Using this method, constitutively active enhancer elements in embryonal stem cells and HeLa cells have been cloned (12, 16, 27, 31). A hormone-regulated enhancer has been isolated by this strategy as well (29). However, a practical restriction of the enhancer trap strategy is the inability to readily distinguish between constitutive and regulated enhancers. We have overcome this impediment and report here the facile identification of regulated enhancers. The enhancer trap strategy we used rests on the ability to select for expression or suppression of the transgene. The vector used contains the fused genes for hygromycin phosphotransferase (Hy) and herpes simplex thymidine kinase (TK). Thus, expression of Hy confers resistance to hygromycin; the positive selection and expression of TK confers sensitivity to ganciclovir, the negative selection. Selection of transfected cells in hygromycin under conditions where the gene should be active followed by selection in ganciclovir under conditions where the gene should be inactive allows the ready detection of regulation. This paper reports that the sequential culture of stably transfected AtT-20 cells in the presence or absence of dexamethasone in combination with the dual selection made possible by the fusion gene (see scheme in Fig. 1) allowed isolation of cell clones containing trapped up- and down-regulated enhancers.

### Materials and Methods

Cell culture reagents and restriction enzymes and molecular biology reagents (except as noted) were obtained from Life Technologies (Grand Island, NY). Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) or Aldrich Chemical Co. (Rouses Point, IL).

#### Construction of the plasmids pHyTKpcl, pHyTKpcs, and pHyTK

The plasmid JA87 was a gift from Jacques Drouin and contained the rat POMC promoter. Full expression and glucocorticoid regulation are conferred by the 513 bp 5' of the transcription start site (48). PCR was used to amplify the promoter fragment from -523 to +10 using the primers: left, 5'-GCACATTCCCTCGAGCCTCTGTTGTCTCCCTTCTC-3' (*XhoI*); and right, 5'-AGGCTTTTCAGCTAGCTCCGTCGCTCCCGTTTGG-3' (*NheI*); these contained the indicated restriction sites (recognition sequence *italicized*). The PCR product was purified by gel electrophoresis and cloned into the TA cloning vector pCRII (Invitrogen, San Diego, CA). A smaller POMC promoter fragment containing bases -109 to +10 and lacking intrinsic promoter activity (48) was prepared using the same right primer and 5'-GCACATTCCCTCGAGGCCGGG-GATTCGCTTGTTC-3' as the left primer. These plasmids were named pCRIIpcl and pCRIIpcs for the large (-523 to +10) and small (-109 to +10) POMC promoter and used to provide the elements to be cloned into tgCMVHyTK. Plasmid tgCMVHyTK (donated by Steve Lupton) contains the structural genes for hygromycin phosphotransferase (Hy) and herpes simplex thymidine kinase (TK), which are expressed as a fusion product driven by the cytomegalovirus (CMV) promoter (30). The CMV promoter in the tgCMVHyTK vector was removed by digestion at the unique *NheI* and *XhoI* sites that form the 5'- and 3'-borders of the CMV promoter. The POMC promoter elements were digested from their respective plasmids using *NheI* and *XhoI* and ligated in place of the CMV fragment. The resulting clones were sequenced to confirm that the expected POMC promoter sequence was present and unaltered. The plasmid containing the 533 bp of POMC promoter was designated pHyTKpcl, and the plasmid containing 109 bp of POMC promoter was designated pHyTKpcs. In another case, the staggered ends of the cut tgCMVHyTK plasmid, minus the CMV fragment, were filled in using Klenow enzyme, and the plasmid was recircularized by ligation to

produce the promoterless plasmid, pHyTK. The predicted behavior of the plasmids, when transfected, would be that pHyTKpcl would express the Hy/TK fusion gene and be down-regulated by glucocorticoids similarly to the POMC gene. pHyTK should not be expressed unless it is inserted into an exon, in-frame, distal to an endogenous promoter. Its behavior should then be based on the behavior of the endogenous promoter that drove it. Expression of pHyTKpcs would require that it be activated by an endogenous enhancer, but spacing would not be as critical as with the promoterless plasmid because pHyTKpcs contains a TATA box. It seemed possible that such an activated transgene might exhibit negative glucocorticoid regulation based on the presence of a receptor-binding element at -53 to -74 (48). However, cell clones stably transfected with this plasmid and selected only for hygromycin resistance did not exhibit dexamethasone suppression of the transgene (data not shown).

#### Transfection

To AtT-20/D1 cells ( $10^7$ ) in a 75-cm<sup>2</sup> flask containing 16 ml serum-free DMEM-Ham's F-12 were added 4 ml serum-free medium containing 10  $\mu$ g plasmid DNA in 100  $\mu$ l Lipofectin (Life Technologies). Twenty-four hours later, newborn calf serum and medium were added to produce a total volume of 100 ml, and the cells were divided into four flasks. Forty-eight hours after the addition of DNA, selection was performed as described in Fig. 1.

#### Cell culture

Routine cell culture and soft agar cloning were performed as previously described (13, 32). Serum-free medium consisted of DMEM-Ham's F-12 (Life Technologies) containing 5  $\mu$ g/ml insulin, 5  $\mu$ g/ml transferrin, and 25 ng/ml selenium (Sigma/Aldrich). Cells were enumerated using a hemocytometer or Coulter counter (Coulter Electronics, Hialeah, FL), and cell viability was determined by exclusion of the vital dye, trypan blue.

### Enhancer Selection Sequence

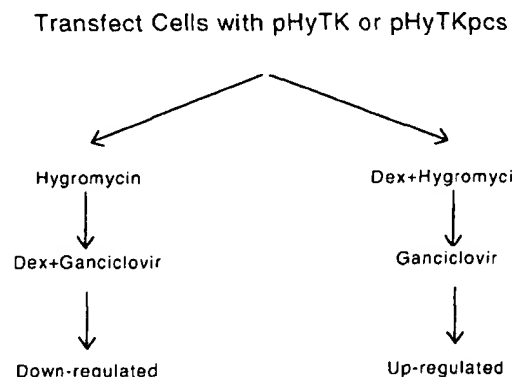
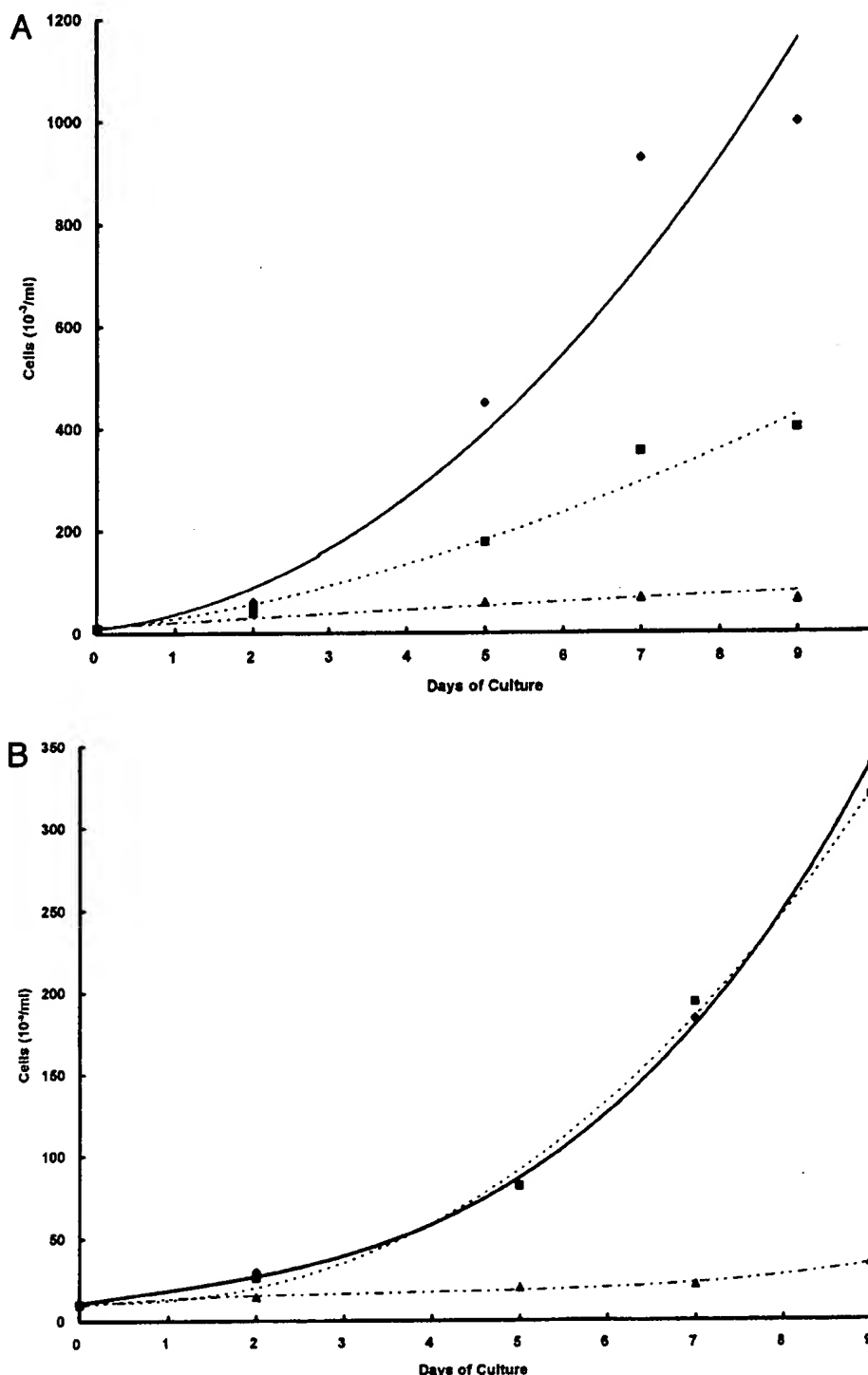


FIG. 1. Experimental sequence used to isolate cell clones with trapped enhancers. Lipofectin-mediated transfection was performed using 10  $\mu$ g plasmid DNA and  $10^7$  AtT-20/D1 cells. To isolate down-regulated enhancers, hygromycin (500  $\mu$ g/ml) was added after 24 h of growth, and the cultures were incubated until vigorously growing clumps of cells were plentiful (10-14 days). The medium was then replaced, and the cells were grown for a day without hygromycin before being placed in medium containing  $10^{-6}$  M dexamethasone; after culture in dexamethasone for 2-4 days to permit complete suppression of enzymatic activity, ganciclovir was added (5  $\mu$ M), and cloning in soft agar was performed after the appearance of vigorously growing clumps of cells (10-14 days). Up-regulated enhancers were trapped over a similar time frame using the sequence as shown and the same concentrations of hygromycin, dexamethasone, and ganciclovir as those used for the down-regulated enhancer isolation.

FIG. 2. a, Growth of AtT-20/PCL3. Cells were plated at an initial density of  $10^4$  cells/ml in untreated medium (no Rx) or medium with 500  $\mu$ g/ml hygromycin (Hy),  $10^{-6}$  M dexamethasone (Dex), 5  $\mu$ M ganciclovir (gc), dexamethasone and hygromycin (dex + hy), or, dexamethasone + ganciclovir (dex + gc). In the case of dexamethasone treatment, cells were treated with dexamethasone for 48 h before the initial plating. At the indicated times, cells were pipetted into a diluent and counted using a Coulter counter. The results depicted are typical of five or six separate experiments. Each point is the mean value from three separate wells. In both panels, one curve is drawn for no Rx, Hy, and Dex ( $\diamond$ ); Dex + Hy, and gc ( $\blacktriangle$ ); and Dex + gc ( $\blacksquare$ ). b, AtT-20/NET1 cells. Cells were plated at an initial density of  $10^4$  cells/ml in untreated medium (no Rx) or medium with 500  $\mu$ g/ml hygromycin (Hy),  $10^{-6}$  M dexamethasone (Dex), 5  $\mu$ M ganciclovir (gc), dexamethasone and hygromycin (dex + hy), or dexamethasone and ganciclovir (dex + gc). In the case of dexamethasone treatment, cells were treated with dexamethasone for 48 h before the initial plating. At the indicated times, cells were pipetted into a diluent and counted using a Coulter counter. The results depicted are typical of five or six separate experiments. Each point is the mean  $\pm$  SE from three separate wells. In both panels, one curve is drawn for no Rx, Hy, and Dex ( $\diamond$ ); Dex + Hy, and gc ( $\blacktriangle$ ); and Dex + gc ( $\blacksquare$ ).



#### RNA electrophoresis and Northern blot analysis

Total RNA prepared by the guanidinium acid-phenol (7) method was run on 1.5% agarose gels containing 2% formaldehyde. The sample buffer contained 40  $\mu$ g/ml ethidium bromide, and the 28S and 18S ribosomal RNA bands were used as size markers of 6333 and 2366 bp, respectively. The gels were transferred by blotting onto GeneScreen Plus (NEN Research Products, Boston, MA), using  $20 \times$  SSC (standard saline citrate) (49). Hybridizations were performed for 16–18 h at 42 C in 30 mM Tris (pH 7.5), 50% formamide, 1% SDS, 1 M NaCl, and 10% dextran

sulfate with DNA probes labeled using random primers (10) and washed in  $2 \times$  SSC-1% SDS and  $0.2 \times$  SSC-1% SDS at 65 C.

#### PCR

PCR was performed using the standard buffer and *Taq* polymerase supplied by Perkin-Elmer Corp. (Norwalk, CT). The 50- $\mu$ l reactions contained 5 pg plasmid DNA or 200 ng genomic DNA and 20 pM of each primer. Reactions were run on a Hybaid Thermal Cycler, and a "hot



start" was employed in which the polymerase addition was delayed until the mixture was heated to 95 C for 2 min. Extension was conducted at 72 C for 2 min (except for the final 5-min extension), and denaturation was performed at 95 C for 60 sec. The initial annealing temperature was 60 C for 2 cycles. This was dropped by 1 C every 2 cycles until 55 C was reached. Then 20 cycles were run using the 55 C annealing temperature. The reactions (10  $\mu$ l) were electrophoresed on 1.0% agarose gels and stained with ethidium bromide.

## Results

### *Selection of cells transfected using the intact promoter-driven HyTK*

Transfection of cells with the pHyTKpcl plasmid containing the HyTK gene driven by the functional (−523 to +10) POMC promoter allowed the selection process for down-regulated enhancers to be tested using a promoter that would be predictably down-regulated by dexamethasone. Cells containing the negatively regulated transgene, pHyTKpcl, were isolated by culture in hygromycin alone, followed by culture in dexamethasone plus ganciclovir and then cloning as described for selection of down-regulated enhancers in Fig. 1. The growth of one clonal line, designated AtT-20/PCL3, was further evaluated under culture conditions of 1) no treatment, 2) treatment with dexamethasone, 3) treatment with hygromycin, 4) treatment with ganciclovir, 5) treatment with dexamethasone and hygromycin, and 6) dexamethasone and ganciclovir (Fig. 2A). Over 9 days, AtT-20/PCL3 cells grew similarly when untreated, when treated with dexamethasone only, or when treated with hygromycin only. Because the curves were so similar, only one line was drawn for all three conditions (Fig. 2A,  $\blacklozenge$ ). The cells did not grow in the presence of dexamethasone and hygromycin or in the presence of ganciclovir alone ( $\blacktriangle$ ). However, growth in dexamethasone and ganciclovir ( $\blacksquare$ ) approached that in untreated medium (2.5-day doubling time *vs.* 1.75 days for untreated cells). These results show that when the fusion transgene was driven by a promoter of known characteristics, it conferred the expected cellular phenotype (Table 1). Expression of the gene resulted in the phenotype of hygromycin resistance and ganciclovir sensitivity, consistent with a constitutive promoter or a down-regulated promoter (Table 1). Suppression of transcription by dexamethasone significantly reduced sensitivity to ganciclovir and increased sensitivity to hygromycin. These results indicated that use of positive and negative selections would allow the detection of stably transfected cell lines in which the fusion HyTK gene was driven by a dexamethasone-regulated enhancer.

### *Selection of enhancer-trapped cells*

AtT-20 cells were transfected with pHyTKpcs (−109 to +10 POMC promoter), and clones were selected as described (Fig. 1) for the trapping of down-regulated enhancers. As the promoter fragment used is insufficient to confer expression (48), expression of the transgene would require that the promoter be influenced by an endogenous enhancer. Two unique cell clones were obtained as determined by Southern blot analysis. The growth of one such clone, AtT-20/NET1, under conditions identical to those used for the AtT-20/PCL3 growth curve, showed very similar results (Fig. 2B).

**TABLE 1.** Expected growth behavior of transfected cells under various culture conditions

Enhancer type	Hygromycin	Ganciclovir	Dex + Hy	Dex + Gc
Constitutive	Grow	No grow	Grow	No grow
Dex up-regulated	No grow	Grow	Grow	No grow
Dex down-regulated	Grow	No grow	No grow	Grow

The doubling times of untreated cells, cells treated with hygromycin alone, or cells treated with dexamethasone alone were very similar to that of the AtT-20/PCL3 clone tested. Moreover, cells treated with dexamethasone and ganciclovir grew as well as the controls, suggesting that dexamethasone suppression might be more complete in these cells *vs.* AtT-20/PCL3 cells. As expected for down-regulation (Table 1, *last line*), cell growth was arrested in the presence of dexamethasone and hygromycin or in the presence of ganciclovir alone.

Cell clones with trapped, positively regulated enhancers were also produced by transfection with the promoterless plasmid (pHyTK) and selection using the strategy for up-regulated enhancers shown in Fig. 1. Thus, three types of cell lines have been produced. Cells designated AtT-20/PCL contain the fusion gene driven by the intact POMC promoter. Cells designated AtT-20/NET contain the fusion gene driven by an endogenous enhancer that is down-regulated by dexamethasone. Lastly, cells designated AtT-20/PET contain the fusion gene driven by an endogenous promoter that is positively regulated by dexamethasone.

### *Unique cell clones identified by PCR*

A PCR strategy was used to determine duplicate cell clones with unique transgene insertion sites. As the plasmid used in the transfection was incompletely linearized, many clones contained various lengths of plasmid backbone 5' of the minimal promoter. Thus, PCR analysis was performed using a right primer within the truncated promoter and left primers at various distances on the plasmid backbone that could have been present 5' of the promoter. PCR of the tgCMVHyTK plasmid with these primers yielded the 472-, 873-, 1480-, and 1837-bp fragments anticipated based on the primer locations. Analysis of the stably transfected cells would yield 472- to 1837-bp fragments depending on the amount of plasmid backbone 5' of the truncated promoter. Analysis of AtT-20/NET1 DNA yielded all four bands, indicating that more than 1837 bp of backbone was present (Fig. 3). However, PCR of AtT-20/NET8 only resulted in 472- and 873-bp bands, indicating that less than 1480 bp of plasmid backbone was 5' of the promoter. These results indicate that these two cell clones are due to different insertion events, although they do not prove that the insertions occurred in different DNA regions.

### *Effects of dexamethasone on hygromycin resistance*

The sensitivities of selected cell clones to hygromycin were tested by growth at various hygromycin concentrations (Fig. 4). After 5 days of growth, AtT-20/NET1 cell growth in 500  $\mu$ g/ml hygromycin was approximately 90% that in the untreated control culture, whereas cell growth in 1000  $\mu$ g/ml

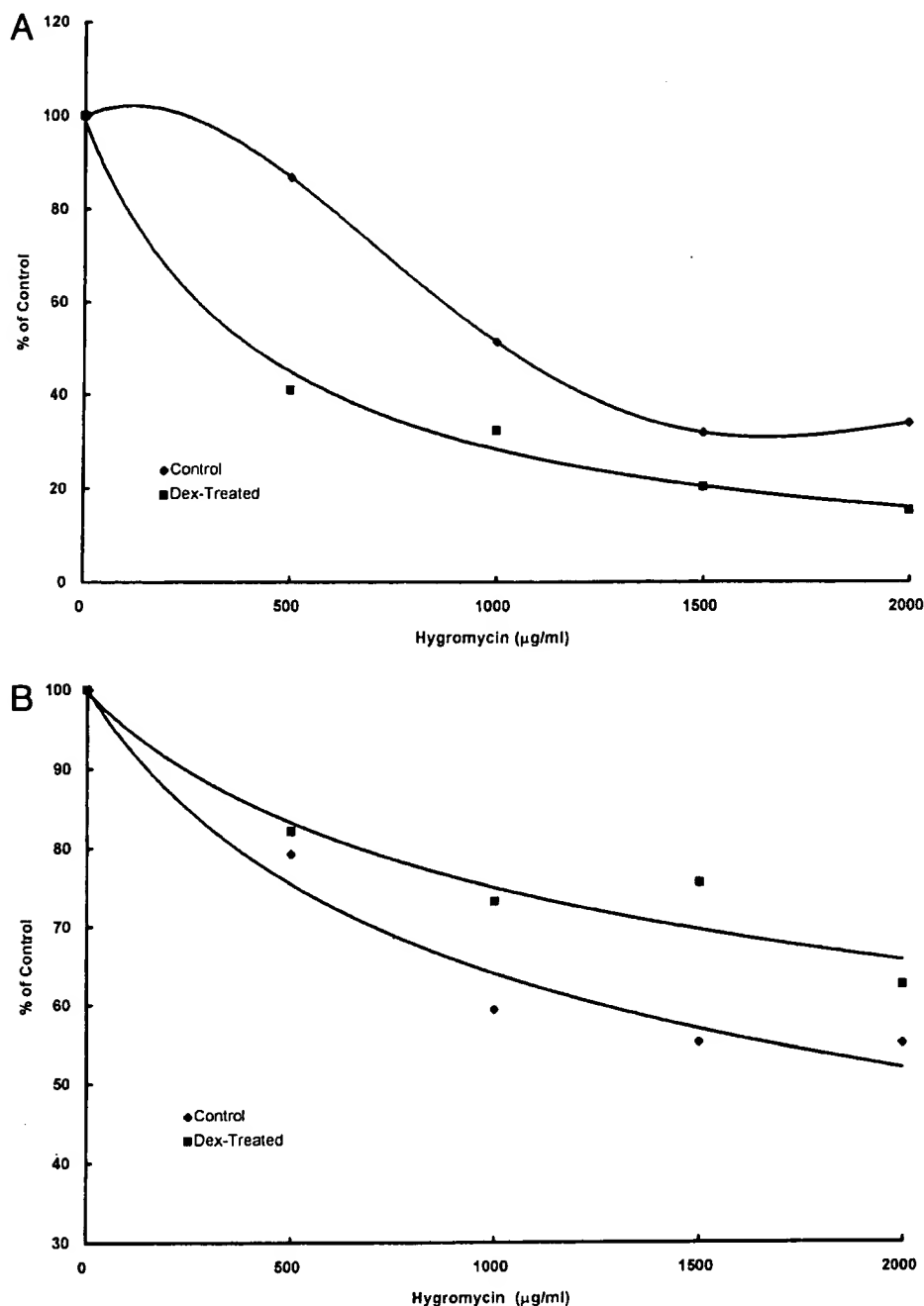


FIG. 3. Effect of dexamethasone on AtT-20/NET1 cell (a) or AtT-20/PET9 cell (b) sensitivity to hygromycin. Cells were cultured for 5 days in 0-2000 µg/ml hygromycin with or without  $10^{-6}$  M dexamethasone. One of 10-15 typical experiments is shown. Triplicate wells were counted, and the mean values were plotted.

hygromycin was only approximately 60% of the control value (Fig. 4a). Treatment with dexamethasone reduced these levels to approximately 40% and 30%, respectively. These results are consistent with the results shown in Fig. 2b after 5 days of culture (see no Rx and Dex + hygromycin).

On the other hand, in the absence of dexamethasone, AtT-20/PET9 cells (Fig. 4b) were more sensitive to hygromycin than AtT-20/NET1 cells, but less sensitive than untransfected AtT-20 cells (not shown). In contrast to AtT-20/NET1, resistance to hygromycin was increased by dexamethasone treatment. These results indicate that there is some basal activity in AtT-20/PET1 cells that is enhanced by dexamethasone.

#### *Transgene messenger RNA (mRNA) is regulated by dexamethasone*

RNA was isolated from clonal cell lines with and without prior treatment with dexamethasone and analyzed by Northern blot. In each case a single major band was seen, and regulation of the transgene could be predicted from the growth behavior of the cell clones. Analysis of AtT-20/PCL3 RNA showed that dexamethasone treatment caused a substantial reduction in HyTK mRNA (Fig. 5, middle panel). Basal HyTK mRNA in AtT-20/NET1 cells was less than that seen with AtT-20/PCL3 cells, and suppression was more complete in AtT-20/NET1 cells (Fig. 5, left panel). Analysis of

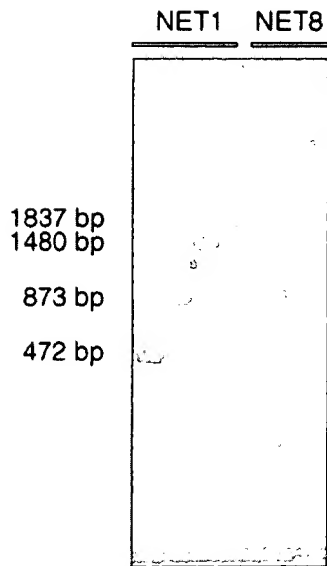


FIG. 4. Agarose gel of PCR products from two enhancer trap cell clones. PCR was performed using a single right primer from within the tgHyTKpcs promoter. The left primers corresponded to positions along the plasmid sequences that could have been left 5' of the promoter after breakage of this plasmid backbone. Four left primers were selected to yield products of 472, 873, 1480, and 1837 bp. Four separate PCR reactions were performed, and the products were separated on a 1% agarose gel and stained with ethidium bromide. NET1, The left four lanes are the results obtained using AtT-20/NET1 DNA. NET8, The right three lanes are the results obtained using AtT-20/NET8 DNA (the third NET8 lane is empty).

AtT-20/PET9 RNA showed that basal expression was low and increased after dexamethasone treatment. The AtT-20/PCL3 and AtT-20/NET1 cell lines yielded a mRNA of about 3200 bp, which was consistent with the predicted size of the HyTK transcript. The AtT-20/PET9 cell line yielded a larger mRNA. As this transgene lacked any promoter elements, the large size probably resulted from integration into an exon, thereby generating a larger mRNA, as previously described for promoter traps (12).

### Discussion

Glucocorticoids affect almost all tissues and may regulate as many as 300 genes in the mouse genome (20) through basic mechanisms that are well understood in principle (9, 43, 50). However, translation of this basic understanding into physiologically useful terms has been limited because only a relatively few glucocorticoid-regulated genes are known. Various strategies, such as differential hybridization (2, 4) and giant two-dimensional gel electrophoresis (36, 55), have been employed to identify glucocorticoid-regulated genes or gene products. The results contained in this paper show that a modified enhancer trap strategy can also be used to readily detect glucocorticoid-regulated enhancers and, thus, determine the genes that define the glucocorticoid response in a given cell line.

Every enhancer trap strategy depends on the selection of cells containing the trapped enhancer. The sequential selection made possible by the HyTK fusion allowed the ready

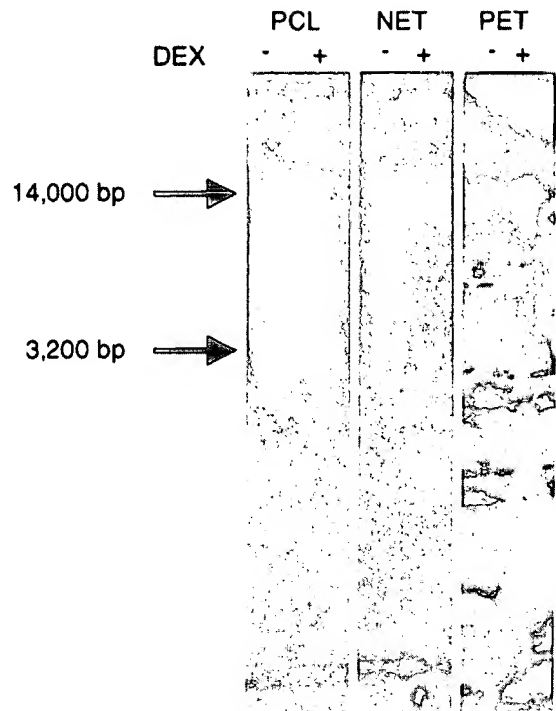


FIG. 5. Northern blot analysis of HyTK regulation in transfected cells. After culture with (+) or without (-)  $10^{-6}$  M dexamethasone for 4 days, cells were harvested, and total RNA was extracted by the acid-phenol method (7). After electrophoresis of 10  $\mu$ g total RNA on 1.5% agarose gels containing 2% formaldehyde, the gels were blotted onto GeneScreen Plus and probed using labeled tgCMVHyTK plasmid. Results are shown from experiments using AtT-20/PCL3 (PCL), AtT-20/NET1 (NET), and AtT-20/PET9 (PET). Visualization of the ethidium bromide-stained samples and reprobing of the filters with a  $\beta$ -actin probe showed that the dexamethasone-treated and untreated lanes contained equal amounts of RNA. The results shown are typical of two or three experiments yielding similar results.

identification of a regulated enhancer, as illustrated when the selection was performed using the transgene driven by the intact POMC promoter (pHyTKpcl). The flexibility of the method is indicated by the selection of up-regulated as well as down-regulated enhancers. Theoretically, any regulated enhancer can be selected for using this strategy and, in fact, thyroid hormone-regulated enhancers have been identified in a cultured cell line using the strategy outlined in this paper (data not shown). Thus, an attractive feature of the strategy is its ease and potential applicability to many regulatory systems. Our analysis suggests that at least 2 up-regulated cell clones and 2 down-regulated ones contained transgenes inserted into dissimilar locations. The lengthy culture times made direct isolation of individual clones impractical, and only 5-30 colonies were selected from soft agar cloning to arrive at the NET and PET clones described here. Thus, the number of unique clones reported does not represent the total number of detectable enhancers present in the AtT-20/D1 cell.

Although AtT-20/NET1 contained a truncated POMC promoter, its growth behavior and mRNA response to dexamethasone were different from those of the intact POMC promoter. The growth of cells transfected with HyTK driven by the intact promoter (AtT-20/PCL3) was slightly slowed in

the presence of dexamethasone and ganciclovir, suggesting incomplete suppression of the HyTK mRNA. This was confirmed by Northern blot analysis and is consistent with previous studies of POMC regulation (17, 34, 48). On the other hand, AtT-20/NET1 cells treated with dexamethasone and ganciclovir grew as well as controls, *i.e.* better than AtT-20/PCL3 cells under the same conditions. Northern blot analysis confirmed that suppression of HyTK mRNA was more complete in AtT-20/NET1 cells than in AtT-20/PCL3 cells. Although additional structural analysis is needed, these results suggest that the enhancer trapped in AtT-20/NET1 cells may not be of POMC origin. Thus, although the number of genes regulated by glucocorticoids in AtT-20/D1 cells is uncertain, these results suggest that several regulated genes exist in addition to POMC because positively regulated enhancers have been identified. However, it will be necessary to isolate additional cells clones and physically identify the DNA sequences encoding glucocorticoid regulation before the minimum number of additional enhancers is known.

Further experience will show whether this strategy has advantages over other gene identification schemes such as subtraction hybridization or giant two-dimensional gel electrophoresis. For instance, in subtraction hybridization, complementary DNA libraries must be prepared for each regulatory condition, whereas in the enhancer trap method, the initial selection step is relatively labor free, because transfection, cell growth under selection conditions, and soft agar cloning are not labor-intensive. At present, the enhancers detected by us are being isolated, a process facilitated by the transgene's presence. Rapid analysis of the DNA flanking sequences should be possible by several methods of genomic walking (1, 25, 40-42). Alternatively, it may be possible to isolate a plasmid-genomic DNA hybrid that can be recircularized and amplified in bacteria. Lastly, as a promoter trap, pHyTK could almost certainly be improved. Friedrich and Soriano (12) found that adding a splice acceptor site to the 5'-end of a promoterless reporter gene dramatically improved trap efficiency. They concluded that most insertions near the 5'-end of genes were into introns, an event favored by the tendency of exons near the 5'-end of genes to be relatively short (22). Adding a splice acceptor site to pHyTK would bring the HyTK marker into closer proximity to the promoter and facilitate identification of the promoter.

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